Stimulation of Suicidal Erythrocyte Death by α -Lipoic Acid

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Key Words

Phosphatidylserine • Scrambling • Calcium • Cell volume • Eryptosis • Apoptosis

Abstract

α -lipoic acid, a nutrient with both, antioxidant and oxidant activity induces apoptosis in a variety of cells. Owing to its proapoptotic potency α -lipoic acid has been suggested for the therapy of cancer. α -Lipoic acid stimulates apoptosis by induction of oxidative stress and subsequent activation of caspases. Oxidative stress could similarly trigger caspase activation and suicidal erythrocyte death or eryptosis, which is characterized by cell membrane scrambling and cell shrinkage. Eryptosis is triggered by increase of cytosolic Ca^{2+} concentration and/or ceramide formation. The present study explored whether α -lipoic acid influences eryptosis. Cell membrane scrambling was estimated from binding of annexin V to phosphatidylserine at the erythrocyte surface, cell volume from forward scatter in FACS analysis, cytosolic Ca^{2+} concentration from Fluo3 fluorescence, caspase activation and ceramide formation utilizing respective antibodies, cytosolic ATP concentration from a luciferase-assay. Within 48 hours, exposure

to α -lipoic acid (10 - 75 mM) significantly decreased forward scatter, increased cytosolic Ca^{2+} concentration, decreased ATP concentration, activated caspase 3, stimulated formation of ceramide and triggered annexin V-binding. Glucose depletion (48 h) was followed by decrease of forward scatter and increase of annexin V-binding, effects significantly augmented in the presence of α -lipoic acid (20 mM). Oxidative stress (30 min 0.3 mM tert-butylhydroperoxide) similarly triggered annexin binding, an effect slightly but significantly blunted by α -lipoic acid. In conclusion, α -lipoic acid triggers eryptosis but by the same token counteracts eryptosis during oxidative stress. α -lipoic acid sensitive eryptosis may lead to anemia and derangements of microcirculation.

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Introduction

α -lipoic acid, a component of vegetables and meat [1] has been used as an antioxidant for the treatment of a variety of diseases, including liver cirrhosis, heavy metal poisoning, and diabetic polyneuropathy [2]. α -lipoic acid

may, however, exert both, antioxidant and oxidant activity [2-6]. In part due to its oxidant activity, α -lipoic acid triggers apoptosis in a variety of cells [4-6]. Accordingly, α -lipoic acid has been suggested for the therapy of cancer [2, 3, 5, 6]. The signaling involved in the triggering of α -lipoic acid induced apoptosis includes the tensin homologue deleted on chromosome 10 (PTEN)/Akt pathway [2, 6], upregulation of poly(ADP-ribose) polymerase (PARP) [3], activation of caspases [2, 3, 5, 6], increase of intracellular Ca^{2+} concentration [3], p53 activation [2], increased expression of Bax [2], down-regulation of Bcl-2 [5], release of cytochrome c from mitochondria [2], downregulation of survivin [2], as well as induction of pro-apoptotic JNK signaling [2].

In analogy to the apoptosis of nucleated cells erythrocytes may undergo suicidal cell death or eryptosis, which is characterized by cell membrane scrambling and cell shrinkage [7]. Eryptosis is triggered by an increase of cytosolic Ca^{2+} concentration secondary to Ca^{2+} entry through Ca^{2+} -permeable cation channels [8-16]. An increase of cytosolic Ca^{2+} concentration leads to activation of Ca^{2+} -sensitive K^{+} channels [17, 18] with subsequent cell shrinkage due to exit of KCl together with osmotically obliged water [19]. Increased Ca^{2+} concentration further stimulates cell membrane scrambling with exposure of phosphatidylserine at the cell surface [16, 20-23]. The erythrocytes are sensitized to the scrambling effect of Ca^{2+} by ceramide [24]. Eryptosis is further triggered by energy depletion, an effect at least partially due to activation of protein kinase C [25]. Erythrocytes express caspases, which could be activated by oxidative stress and in turn stimulate cell membrane scrambling [22, 26]. Ca^{2+} induced eryptosis does, however, not require activation of caspases [20, 24, 27].

The present study explored, whether α -lipoic acid may trigger eryptosis and, if so, to identify underlying mechanisms.

Materials and Methods

Erythrocytes, solutions and chemicals

Leukocyte-depleted erythrocytes were kindly provided by the blood bank of the University of Tübingen. The study is approved by the ethics committee of the University of Tübingen (184/2003V).

Erythrocytes were incubated *in vitro* at a hematocrit of 0.4% in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO_4 , 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 CaCl_2 ; pH 7.4 at 37°C for 48 hours. Where indicated, α -lipoic acid (T1395, Sigma, Freiburg, Germany) was

added at the indicated concentrations. In Ca^{2+} -free Ringer, 1 mM CaCl_2 was substituted for 1 mM ethylene glycol tetraacetic acid (EGTA).

FACS analysis of annexin V-binding and forward scatter

After incubation under the respective experimental condition, 50 μl cell suspension were washed in Ringer solution containing 5 mM CaCl_2 and then stained with Annexin-V-Fluos (1:500 dilution; Roche, Mannheim, Germany) in this solution for 20 min under protection from light. In the following, the forward scatter (FSC) of the cells was determined, and annexin V fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS calibur (BD, Heidelberg, Germany).

Measurement of intracellular Ca^{2+}

After incubation 50 μl suspension of erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Calbiochem, Bad Soden, Germany) in Ringer solution containing 5 mM CaCl_2 and 2 μM Fluo-3/AM. The cells were incubated at 37°C for 20 min and washed twice in Ringer solution containing 5 mM CaCl_2 . The Fluo-3/AM-loaded erythrocytes were resuspended in 200 μl Ringer. Then, Ca^{2+} -dependent fluorescence intensity was measured in fluorescence channel FL-1 in FACS analysis.

Measurement of Caspase-3 and Caspase-8 activity

Caspase 8 and Caspase 3 activation was determined using kits from Biovision according to manufacturer's instruction. Briefly 1×10^5 cells were washed twice with cold PBS, fixed and permeabilized with 'Cytfix/Cytoperm' solution and then by washing twice with 'Perm/Wash' buffer. Then cells were stained with FITC conjugated anti-active Caspase 8 or Caspase 3 antibody in 'Perm/Wash' buffer for 60 minutes. After 2 washing steps, the cells were analyzed by flow cytometry (FACS Calibur, BD Biosciences).

Determination of ceramide formation

To determine ceramide abundance at the cell surface, a monoclonal antibody-based assay was used. After incubation, cells were stained for 1 hour at 37°C with 1 $\mu\text{g}/\text{ml}$ anti-ceramide antibody (clone MID 15B4; Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) at a dilution of 1:5. After two washing steps with PBS-BSA, cells were stained for 30 minutes with polyclonal fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG and IgM specific antibody (Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA. Unbound secondary antibody was removed by repeated washing with PBS-BSA. Samples were then analysed by flow cytometric analysis in FL-1.

Determination of intracellular ATP concentration

For determination of erythrocyte ATP, 90 μl of erythrocyte pellets were incubated for 48 h at 37°C in Ringer solution with or without α -lipoic acid (final hematocrit 5%). All manipulations were then performed at 4°C to avoid ATP degradation. Cells were lysed in distilled water, and proteins were precipitated by

Fig. 1. Effect of α -lipoic acid on cytosolic Ca^{2+} concentration in erythrocytes. A. Histogram of Fluo3 fluorescence in a representative experiment of erythrocytes exposed for 48 hours to Ringer solution without (-, black line) and with (+, red line) 50 μM α -lipoic acid. B. Arithmetic means $\pm$ SEM ($n = 5$) of the geo means (geometric mean of the histogram) of Fluo3 fluorescence in erythrocytes exposed for 48 hours to Ringer without (white bar) or with (black bars) α -lipoic acid. *, *** indicate significant difference ($p < 0.05$, $p < 0.001$) from the respective value in the absence of α -lipoic acid (ANOVA).

addition of HClO_4 (5%). After centrifugation, an aliquot of the supernatant (400 μl) was adjusted to pH 7.7 by addition of saturated KHCO_3 solution. After dilution of the supernatant, the ATP concentrations of the aliquots were determined utilizing the luciferin-luciferase assay kit (Roche Diagnostics) on a luminometer (Berthold Biolumat LB9500, Bad Wildbad, Germany) according to the manufacturer's protocol. ATP concentrations are expressed in mmol/l cytosol of erythrocytes.

Statistics

Data are expressed as arithmetic means $\pm$ SEM. Statistical analysis was made using paired ANOVA with Tukey's test as post-test, as appropriate. n denotes the number of different erythrocyte specimens studied. Since different erythrocyte specimens used in distinct experiments are differently

Fig. 2. Effect of α -lipoic acid on erythrocyte forward scatter. A. Original histogram of the forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (-, black line) and with (+, red line) 50 μM α -lipoic acid. B. Arithmetic means $\pm$ SEM ($n = 5$) of erythrocyte forward scatter following exposure for 48 hours to Ringer solution without (white bar) or with (black bars) α -lipoic acid at the indicated concentrations. *** ($p < 0.001$) indicate significant difference from the respective value without exposure to α -lipoic acid (ANOVA).

susceptible to eryptotic effects, the same erythrocyte specimens have been used for control and experimental conditions.

Results

A major trigger of eryptosis is an increase of cytosolic Ca^{2+} concentration in erythrocytes [28]. Thus, Fluo 3 fluorescence has been utilized to elucidate whether α -lipoic acid influences erythrocyte Ca^{2+} concentration. As shown in Fig. 1, α -lipoic acid exposure increased the Fluo3 fluorescence, indeed pointing to an increase of cytosolic Ca^{2+} concentration.

Fig. 3. Effect of α -lipoic acid on phosphatidylserine exposure of erythrocytes. A. Histogram of erythrocyte annexin V-binding in a representative experiment of erythrocytes exposed for 48 hours to Ringer solution without (-, black line) and with (+, red line) 50 μ M α -lipoic acid, M is a marker indicating phosphatidylserine exposing cells. B. Arithmetic means $\pm$ SEM (n = 5) of the percentage of phosphatidylserine-exposing erythrocytes following exposure for 48 hours to Ringer solution without (white bar) or with (black bars) α -lipoic acid. **, *** (p<0.01, p<0.001) indicate significant difference from the respective value without exposure to α -lipoic acid. C. Arithmetic means $\pm$ SEM (n = 4) of the percentage of phosphatidylserine-exposing erythrocytes following exposure for 48 hours to Ringer solution in the presence (left bars) or absence (right bars) of extracellular Ca^{2+} without (white bars) or with (black bars) 50 μ M α -lipoic acid. *** (p<0.001) indicate significant difference from the respective value without exposure to alpha-lipoic acid. ## (p<0.001) indicate significant difference from the respective value in the presence of extracellular Ca^{2+} (ANOVA). D. Arithmetic means $\pm$ SEM (n = 4) of the percent increase of phosphatidylserine-exposing erythrocytes following 50 μ M α -lipoic acid exposure for 48 hours to that of erythrocytes incubated in Ringer in the presence (black bar) or absence (white bar) of extracellular Ca^{2+} .

An increase of cytosolic Ca^{2+} concentration stimulates K^+ channels with subsequent exit of KCl and cell shrinkage [17-19]. Thus, forward scatter was employed to estimate alterations of cell volume. As illustrated in Fig. 2, exposure of erythrocytes for 48 hours to Ringer solution with α -lipoic acid was indeed followed by a decrease of FSC, an effect reaching statistical significance at ≥ 20 μ M α -lipoic acid concentrations.

An increase in cytosolic Ca^{2+} concentration further stimulates cell membrane scrambling with phosphatidylserine exposure at the cell surface, which could be identified by determination of annexin V-binding. As shown in Fig. 3, the percentage of annexin V binding erythrocytes was markedly increased following exposure of erythrocytes for 48 hours to Ringer solution containing α -lipoic acid (10 μ M to 75 μ M).

Fig. 4. Effect of α -lipoic acid on erythrocyte ATP content. Arithmetic means $\pm$ SEM (n = 4) of the ATP concentration after a 48 hours incubation in Ringer solution without (white bar) or with (black bars) α -lipoic acid at the indicated concentrations. *, ** indicate significant difference (p<0.05, p<0.01) from control (absence of α -lipoic acid) (ANOVA).

Fig. 5. Effect of α -lipoic acid on caspase-3 activity in erythrocytes. A. Histogram of erythrocyte caspase-3 fluorescence in a representative experiment of erythrocytes from healthy volunteers incubated for 48h in Ringer solution without (-, black line) and with (+, red line) 50 μ M α -lipoic acid. B. Arithmetic means $\pm$ SEM (n = 5) of the caspase-3 fluorescence in erythrocytes exposed for 48 hours to Ringer without (white bar) or with (black bars) α -lipoic acid. *, *** indicate significant difference (p<0.05, p<0.001) from the respective value in the absence of α -lipoic acid (ANOVA).

To test whether the cell membrane scrambling effect of α -lipoic acid is accounted for by the increase of cytosolic Ca^{2+} concentration, erythrocytes were exposed to α -lipoic acid in the absence of extracellular Ca^{2+} . As shown in Fig. 3, the stimulation of annexin V binding by α -lipoic acid was significantly blunted in the absence of extracellular Ca^{2+} .

As shown earlier [25], eryptosis could be triggered by energy depletion. Accordingly, additional experiments were performed to determine, whether exposure to α -lipoic acid influences ATP concentrations in erythrocytes. As shown in Fig. 4, a 48 hours exposure of human

Fig. 6. Effect of α -lipoic acid on ceramide formation in erythrocytes. A. Histogram of erythrocyte ceramide content in a representative experiment of erythrocytes from healthy volunteers incubated for 48h in Ringer solution without (-, black line) and with (+, red line) 50 μ M α -lipoic acid. B. Arithmetic means $\pm$ SEM (n = 5) of the geo means (geometric mean of the histogram) of ceramide in erythrocytes exposed for 48 hours to Ringer without (white bar) or with (black bars) α -lipoic acid. *** indicate significant difference (p<0.001) from the respective value in the absence of α -lipoic acid (ANOVA).

erythrocytes to α -lipoic acid (=75 μ M) significantly reduced the cellular ATP concentration.

To test whether the stimulation of cell membrane scrambling by α -lipoic acid exposure is paralleled by caspase activation, erythrocytes were exposed to α -lipoic acid and caspase-3 and caspase-8 activity determined by flow cytometry. As shown in Fig. 5, the treatment with α -lipoic acid caused a significant increase in caspase-3 activity in erythrocytes, while there was no effect on caspase-8 activity (data not shown).

As eryptosis is similarly stimulated by ceramide [24], the effect of α -lipoic acid on ceramide formation in

Fig. 7. Effect of α -lipoic acid on erythrocyte forward scatter following glucose depletion. A. Original histogram of the forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (-, black line) and with (+, red line) 50 μ M α -lipoic acid in the absence of glucose. B. Arithmetic means $\pm$ SEM ($n = 5$) of erythrocyte forward scatter following exposure for 48 hours to Ringer solution without (white bar) or with (black bars) α -lipoic acid at the indicated concentrations in the absence of glucose. *, ** ($p < 0.05$, $p < 0.01$) indicate significant difference from the respective value without exposure to α -lipoic acid (ANOVA).

Fig. 8. Effect of α -lipoic acid on phosphatidylserine exposure of erythrocytes following glucose depletion. A. Original histogram of the annexin V binding of erythrocytes following exposure for 48 hours to Ringer solution without (-, black line) and with (+, red line) 50 μ M α -lipoic acid in the absence of glucose, M is a marker indicating phosphatidylserine exposing cells. B. Arithmetic means $\pm$ SEM ($n = 5$) of erythrocyte annexin V binding following exposure for 48 hours to Ringer solution without (white bar) or with (black bars) α -lipoic acid at the indicated concentrations in the absence of glucose. *** ($p < 0.001$) indicate significant difference from the respective value without exposure to α -lipoic acid (ANOVA).

erythrocytes was evaluated. As a result, α -lipoic acid exposure indeed increased the ceramide formation in erythrocytes (Fig. 6).

Additional experiments were performed to explore whether α -lipoic acid interacts with the response of erythrocytes to glucose depletion [25]. According to forward scatter, exposure of the erythrocytes with glucose free solutions for 48 hours led to pronounced cell shrinkage. The additional treatment with α -lipoic acid

significantly enhanced the effect of glucose depletion on forward scatter (Fig. 7).

Glucose depletion further stimulates cell membrane scrambling and thus annexin V binding. As shown in Fig. 8, the percentage of annexin V binding erythrocytes was markedly increased following glucose depletion, an effect further augmented by the presence of α -lipoic acid.

Additional experiments were performed to explore whether α -lipoic acid interferes with the effect of

Fig. 9. Stimulation of phosphatidylserine exposure by oxidative stress in the presence and absence of α -lipoic acid. A. Histogram of erythrocyte annexin V-binding in a representative experiment of erythrocytes from healthy volunteers incubated for 30 minutes in the presence of tert-butylhydroperoxide (0.3 mM) in the absence (1, black line) and presence (2, red line) of 20 μ M α -lipoic acid. B. Arithmetic means $\pm$ SEM ($n = 5$) of the percentage of annexin V-binding erythrocytes following incubation for 30 minutes in the absence (CON, open bars) or presence (OX-C, closed bars) of tert-butylhydroperoxide (0.3 μ M) in the absence (grey bar) and presence (black bars) of 20 μ M α -lipoic acid. *** ($p < 0.001$) indicate significant difference from the absence of oxidative stress, # ($p < 0.05$) indicate significant difference from the absence of α -lipoic acid (ANOVA).

oxidative stress on erythrocytes. According to annexin V binding, exposure of erythrocytes to 0.3 mM tert-butylhydroperoxide was followed by an increase of phosphatidylserine exposure, an effect again slightly but significantly blunted in the presence of 20 μ M α -lipoic acid (Fig. 9).

Discussion

According to the present observations exposure of human erythrocytes to α -lipoic acid increases cytosolic Ca^{2+} concentration, an effect in turn contributing to or even accounting for the erythrocyte shrinkage, which presumably results from activation of Ca^{2+} sensitive K^{+} channels [17, 18]. The molecular identity of the channels has previously been shown to be SK4 [29], a channel inhibited by TRAM 34 [30]. An increase of cytosolic Ca^{2+} concentration at least partially accounts for stimulation of cell membrane scrambling following α -lipoic acid treatment. Accordingly, the effect of α -lipoic acid on Annexin V binding is significantly blunted in the nominal absence of Ca^{2+} . Removal of Ca^{2+} does, however, not completely abrogate α -lipoic acid induced cell membrane scrambling, i.e. even in the absence of extracellular Ca^{2+} , α -lipoic acid significantly increased the percentage of annexin V binding erythrocytes. Thus, additional mechanisms must be triggered by α -lipoic acid, which could contribute to the stimulation of cell membrane scrambling.

As a matter of fact, α -lipoic acid decreases ATP concentration and enhances ceramide formation, both mechanisms known to stimulate eryptosis [7]. ATP depletion has previously been shown to trigger eryptosis at least partially due to activation of protein kinase C [25]. Ceramide sensitizes the cell to the proeryptotic effects of increased cytosolic Ca^{2+} activity [24]. Ceramide formation is stimulated by platelet activating factor (PAF), which activates a sphingomyelinase leading to the breakdown of sphingomyelin [31]. Accordingly, eryptosis following osmotic shock is blunted by the sphingomyelinase inhibitor 3,4-dichloroisocoumarin, by genetic knockout of PAF receptors (PAF receptor knockout mice), and by the PAF receptor antagonist ABT491 [31].

In view of the antioxidant activity of α -lipoic acid [2-6], its ability to stimulate caspases may be surprising. Erythrocytes express caspases [22, 26], which are activated by oxidative stress [32]. The caspases cleave the anion exchanger band 3 [33] and stimulate phosphatidylserine exposure of erythrocytes [26].

The present observations may have possible implications for the use of α -lipoic acid as dietary supplement and in pharmacotherapy. The substance is used as an antioxidant in the management and for the prevention of several disorders [2, 34-38]. Moreover α -lipoic acid has been considered for the treatment of malignancies [2, 3, 5, 6]. In healthy individuals, the weak stimulating

effect of α -lipoic acid may not be relevant and eryptosis may occur only in toxic dosages. At least in theory, however, the sensitivity to the effects of α -lipoic acid may be enhanced by several disorders and following ingestion of several xenobiotics. Earlier α -lipoic acid has been shown to participate in the pathophysiology of sickle cell anemia [39, 40] which is known to foster eryptosis [41]. Further disorders associated with accelerated eryptosis [7], including iron deficiency [42], phosphate depletion [43], Hemolytic Uremic Syndrome [44], sepsis [45], malaria [41, 46-50], or Wilson's disease [51]. Moreover, a wide variety of xenobiotics and endogeneous substances do stimulate eryptosis [7, 52-64]. All those conditions and chemicals may foster development of anemia [7].

Phosphatidylserine-exposing erythrocytes may further adhere to the vascular wall [65-69] and stimulate blood clotting [65, 70, 71]. Enhanced eryptosis may thus interfere with microcirculation and contribute to the

vascular injury of metabolic syndrome [72].

In conclusion, α -lipoic acid stimulates cell shrinkage and cell membrane scrambling of erythrocytes, two hallmarks of eryptosis, the suicidal death of erythrocytes. The substance does, therefore, not only trigger apoptosis of nucleated tumor cells but is similarly effective in the absence of nuclei and mitochondria.

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